AD NUMBER

AD455886

NEW LIMITATION CHANGE

TO

Approved for public release, distribution unlimited

FROM

Distribution authorized to U.S. Gov't. agencies and their contractors; Administrative/Operational Use; Dec 1964. Other requests shall be referred to Army Biological Labs., Fort Detrick, MD.

AUTHORITY

BDRL ltr, 27 Sep 1971

TECHNICAL MANUSCRIPT 173

PARTIAL PURIFICATION OF THE ORGANISM OF PSITTACOSIS GROWN IN CHICK EMBRYO YOLK SAC

DECEMBER 1964

UNITED STATES ARMY
BIOLOGICAL LABORATORIES
FORT DETRICK

U.S. ARMY BIOLOGICAL LABORATORIES
Fort Detrick, Frederick, Maryland

TECHNICAL MANUSCRIPT 173

PARTIAL PURIFICATION OF THE ORGANISM OF PSITTACOSIS GROWN IN CHICK EMBRYO YOLK SAC

William N. Shirey

Warren G. Dorsey

William C. Patrick, III

Process Development Division
DIRECTOR OF DEVELOPMENT

Project 1C522301A082

December 1964

DDC AVAILABILITY NOTICE

Qualified requestors may obtain copies of this publication directly from DDC.

Foreign announcement and dissemination of this publication by DDC is limited.

ABSTRACT

A procedure described for the partial purification of suspensions of the organism of psittacosis utilizes tryptic digestion, calcium chloride - dextran sulfate precipitation, and centrifugation through a sucrose barrier. More than 50% of the organisms in the original suspension are recovered in 80% of the original volume with approximately 95% of the lipids and 85% of the proteins removed. This method is suggested as a preliminary purification procedure in laboratories desiring relatively large amounts of purified agent for studies on metabolism, physical properties, or antigenicity of the organism. Significant aspects of the investigations leading to the selection of the procedure are included.

I. BACKGROUND

Studies on the physical, chemical, antigenic, and metabolic characteristics of rickettsiae, psittacosis, or similar types of organisms require relatively large quantities of the organism in as pure a form as possible. Many investigators have not considered the maintenance of infectivity essential, but we believe that physicochemical studies conducted with purified preparations are more valid if these preparations preserve, as nearly as possible, the biological activity of the organism. Purification techniques involving fat solvents (ether) that gave satisfactory results with rickettsiae[1,2] are not useful with the psittacosis organism grown in the yolk sac of embryonated chicken eggs when one desires to maintain infectivity. The organism of psittacosis is also more sensitive to trichlorotrifluoroethane (Freon-113*) than the "true" viruses and Coxiella burnetii.[3]

The method of Silverman and Fiset,[4] using cellulose, is reported to be satisfactory but requires several steps in which there are losses in titer during processing, and the final product is low in titer. The method of Comer and Wachter[3] also requires several steps and uses a highly volatile liquid, dichlorotetrafluoroethane (Freon-114*). The knowledge that the organism of psittacosis is closely associated with the lipids in the chicken embryo and the description by Sakagami and Zilversmit[5] of a method for separating lipoproteins from hypercholesterolamic dog serum led us to speculate that organisms might be effectively separated from the extraneous yolk sac lipids by dextran sulfate precipitation. The incorporation of this idea into a workable and useful procedure is the basis for the present paper.

II. MATERIALS AND METHODS

The organism used in these studies is one of the group of psittacosis lymphogranuloma venereum (LVG)[6] isolated from a human case of pneumonitis in Louisiana[7] and designated Borg strain. Seven-day embryonated chicken eggs were inoculated by the yolk sac route with 2000 mouse intracranial 50% lethal doses ($MICLD_{50}$) and then incubated at 37 C for 96 hours. The yolk sac was harvested from those dying between 72 and 96 hours.

---

* Freon Products Div., E.I. DuPont De Nemours & Co., Inc., Wilmington 98, Delaware.

The yolk sacs were harvested into a beaker chilled with ice and all subsequent steps were performed at approximately 4 C. The yolk sacs were blended for 5 minutes in a Waring Blendor. Bacto-trypsin (0.1%) was added to the slurry, which was stored at 4 C for approximately 20 hours, then diluted to the desired concentration with the appropriate diluting fluid. The diluted yolk sacs were mixed with the designated levels of dextran sulfate, calcium chloride, and sodium chloride by hand shaking in a closed bottle.

This mixture was centrifuged in an International centrifuge PR-2 using the 1E277 trunnion and safety cups for designated times and revolutions per minute (rpm) at 4 C.

The concentration of organisms per unit volume in relation to other solids was used as the criterion for judging purity. Solids, lipids, and proteins were analyzed on the more promising fractions.

The solids in the fractions were estimated from 5-ml samples evaporated to constant weight on a Cenco moisture balance.

The lipids were estimated from the amount of ethyl-ether-soluble substances remaining in the ether from four ether extractions evaporated to constant weight at 100 C.

The proteins were estimated by the method of Lowry, et al.[8]

The titer of infective organisms per unit of material was estimated by either the method of J.M. Riley* or that of Reed and Muench.[9] Swiss-Webster strain white mice (10 to 14 grams) were inoculated intracranially with 0.03 ml of the appropriate dilution of organisms.** In using the method of Reed and Muench, 4 tenfold dilutions were inoculated and animals were observed for 14 days postinoculation.

---

* Personal communication, 1963.

** In conducting the research reported herein the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

III. RESULTS

The initial investigation was designed to determine the best combination of diluents, salt concentration, dextran sulfate concentration, and centrifugal conditions to yield the highest titer in the purified product derived from 20% yolk sac. Four diluents, two centrifuge conditions, and four levels each of dextran sulfate, calcium chloride, and sodium chloride were tested. As the level of one chemical was varied the others were held at these final concentrations: sodium chloride, 0.14%; dextran sulfate, 0.34%; and calcium chloride, 1.58%. This design is illustrated in Table 1. We anticipated the settling of the debris and that the organisms would remain suspended under the conditions imposed.

The partially purified or top fractions from the 96 treatment combinations were assayed for titer using mean time to death (MTD) procedures.* The reciprocals of the MTD (in hours) multiplied by the volume of the fraction gave a factor representing the observed total titer in the fraction. Thus, the control titer factors before purification were 159, 154, 145, and 123 for the phosphate, dextrose-skim milk, dextrose, and distilled water diluents respectively. The results were expressed as per cent of the control factor. This factor, although not always meaningful, is satisfactory for screening where the volumes and the titers fall within relatively narrow limits. The results showed that the volume of the fraction generally had more influence on total titer recovered than the level of titer per milliliter. The organisms tended to settle with the lipids and proteins to the bottom of the tubes and thus large numbers were discarded in a small volume. Twenty-one of the 96 combinations of conditions yielded 60% or more of the virus in the clarified fraction. Of these, 14 were obtained using a maximum centrifugal force of 400 x g for 15 minutes. Thirteen were produced with distilled water as a diluting fluid; only one was obtained with a diluting fluid of 7.5% dextrose in skim milk. Except for the 2.8% calcium chloride, the individual levels of sodium chloride, calcium chloride, and dextran sulfate tested did not produce significant differences in the recovery of organisms. The highest level of calcium chloride tested, 2.8%, when combined with milk and dextrose or with dextrose alone, produced a larger volume of centrifugal solids with the lower gravitational force. This resulted in more of the total titer in the discarded fraction.

Generally, the combination of calcium chloride and dextran sulfate resulted in better separation of the sedimentable solids from the yolk sac suspension than was achieved from nontreated yolk sacs. The organisms however, settled with the inert solids during centrifugation.

---

* Riley, Personal communication, 1963.

TABLE 1. DESIGN OF EXPERIMENT TO TEST EFFECTS OF CONCENTRATION OF CHEMICALS, CENTRIFUGATION CONDITIONS, AND DILUENT[a] ON RECOVERY OF THE ORGANISM OF PSITTACOSIS

Centrifuge Conditions		1[b]			2[b]			3[b]		
rpm	time, min	NaCl %	CaCl %	DS[c] %	NaCl %	CaCl %	DS[c] %	NaCl %	CaCl %	DS[c] %
1000	5	0.14	1.58	0.34	0.14	0.36	0.34	0.14	1.58	0.17
		0.29	1.58	0.34	0.14	0.72	0.34	0.14	1.58	0.34
		0.57	1.58	0.34	0.14	1.58	0.34	0.14	1.58	0.69
		0.86	1.58	0.34	0.14	2.87	0.34	0.14	1.58	1.38
1500	10	0.14	1.58	0.34	0.14	0.36	0.34	0.14	1.58	0.17
		0.29	1.58	0.34	0.14	0.72	0.34	0.14	1.58	0.34
		0.57	1.58	0.34	0.14	1.58	0.34	0.14	1.58	0.69
		0.86	1.58	0.34	0.14	2.87	0.34	0.14	1.58	1.38

a. A 20% YS suspension was prepared in each of the following diluents for use with the above chemical combinations and centrifugation conditions:

1. 0.25 gram per liter KH_2PO_4, 2.5 grams per liter Na_2HPO_4, and 0.1% monasodium glutamate in distilled water.
2. Pasteurized fresh skim milk plus 7.5% dextrose.
3. 7.5% dextrose in distilled water.
4. Distilled water.

b. Chemical combination.
c. Dextran sulfate.

On the assumption that psittacosis organisms have a higher density than most lipoproteins, density barrier centrifugation was explored, using various concentrations of sucrose along with calcium chloride and dextran sulfate to enhance the separation of the particulate material. This experiment was performed with a 50% yolk sac suspension in distilled water treated with 1.58% calcium chloride, 0.14% sodium chloride, and 0.34% dextran sulfate layered over 10, 13, 16, 19, and 22% sucrose solutions and followed by centrifugation at 2000 x g for 30 minutes. The results were evaluated on the basis of the ratio of the volume of nonspecific solids (top) to that of the purified layer (bottom) and the disposition of the organisms (Table 2). The 10% sucrose solution allowed a large fraction of the sedimentable solids and organisms to settle to the bottom with little separation of the organisms from the mixture. The 22% sucrose prevented the settling of essentially all yolk sac solids, including the organisms that remained in the upper complex mixture. Note in Table 2 that the concentration of sucrose in the bottom layer only must be considered in this evaluation and the yolk sac component is additive to the sucrose concentration.

TABLE 2. EFFECT OF CONCENTRATION OF SUCROSE ON SEDIMENTATION OF ORGANISM OF PSITTACOSIS TREATED WITH CALCIUM CHLORIDE, DEXTRAN SULFATE, AND CENTRIFUGATION

Sucrose Concentration, %	Fraction After Centrifugation		Volume, ml	Total Solids Per ml, %	Yolk Sac Solids Per ml, %	Titer, $\log_{10}$ $MICLD_{50}$/ml
	Top	Bottom				
10	X		160	6.0	6.0	6.8
		X	34	12.1	2.1	6.9
13	X		57	17.5	17.5	7.9
		X	143	13.2	0.2	7.4
16	X		60	18.2	18.2	8.2
		X	140	16.1	0.1	6.9
19	X		65	18.4	18.4	8.0
		X	135	19.4	0.4	6.7
22	X		62	19.0	19.0	8.7
		X	138	21.3	0.0	6.5
Control[a/]				25.2	25.2	7.8

a. Control: 50% YS with no treatment.

The 13 and 16% sucrose stopped a large portion of the sedimentable solids but allowed the organisms to pass into the sucrose layer. Thus 16% sucrose was chosen as the concentration for future study because it produced a good separation of organisms from the other yolk sac components and a large percentage of the organisms settled into the relatively clear sucrose layer.

The foregoing information was used to establish a purification procedure that was tested on eight occasions to determine the reliability of the procedure and to characterize the product. This procedure was: (i) for each milliliter of a trypsinized 50% yolk sac solution in distilled water add 0.2 ml of 2% NaCl, 0.10 ml of 5% $CaCl_2$, and 0.096 ml of 2.5% sodium dextran sulfate solution, giving final concentrations of 0.29, 0.36, and 0.17% respectively; (ii) mix the reagents with the yolk sac suspension by hand shaking in a stoppered bottle; (iii) layer the mixture over 16% sucrose in a ratio of three parts mixture to two parts sucrose solution; (iv) centrifuge at 2000 x g for 30 minutes at 4 C; (v) decant the sucrose layer and assay for titer, protein, lipids, and solids. The titer was calculated by the method of Reed and Muench.[9]

To simplify the presentation of data (Table 3) the raw data have been corrected on the basis of 100 ml of 50% yolk sac being processed.

The levels of organisms in the 50% yolk sac and that in either the discard of the partially purified fractions are not statistically different from one another on a unit-volume basis. The 80-ml volume of the purified fraction in contrast to 127 ml in the discard suggests that 39% of the original organisms were in the purified fraction. On a unit-volume basis the purified product has 5.9 times less protein, 14 times less lipids, and 2.6 times less solids than the original 50% YS. On a total basis, 85.5% of the protein and 94.3% of the lipid were removed in the purification procedure. The final product was collected in 16% sucrose and 55% of the total dry solids of the purified fraction was sucrose.

The purified fraction was concentrated by high-speed centrifugation and the resulting product, diluted 1:60 with distilled water, was examined microscopically. Electron micrographs show particles free of similar-sized extraneous matter that appear to be exclusively the small dense form of the organism (Figure 1).

TABLE 3. BIOLOGICAL AND PHYSICAL PROPERTIES OF FRACTIONS OBTAINED DURING PURIFICATION OF THE ORGANISM OF PSITTACOSIS

Type of Assay	Fractions Assayed		
	50% YS (starting)	Discard	Purified
Titer, $\log_{10}$ $MICLD_{50}$/ml	9.2±0.6	8.9±0.6	9.2±0.6
$\log_{10}$ $MICLD_{50}$ (total)	11.2	11.0	11.1
% original		63[a]	80[a]
Volume, ml	207[b]	127	80
Solids, g/100 ml	11.5±2.85	18.5±3.57	11.8±2.92
total, g	11.5	8.5	9.59
% original		74.0	37.7[c]
Lipids, mg/ml	18.3±6.8	39.9±28.9	1.3±0.90
total, mg	1830	1834.4	104.0
% original		>100	5.7
Proteins, mg/ml	27.3±6.1	34.7±22.7	4.6±3.42
total, mg	2730	1596.2	369.4
% original		58.5	13.5

a. Since small changes in titer below the level of assay precision represent large changes in per cent, these per cent figures cannot be considered different.
b. This volume contains 100 ml of 50% YS plus 40 ml of reagents and 67 ml of 16% sucrose.
c. These solids as measured included 55% sucrose added in the process, thus only 4.34 g of the 9.59 g represent original solids.

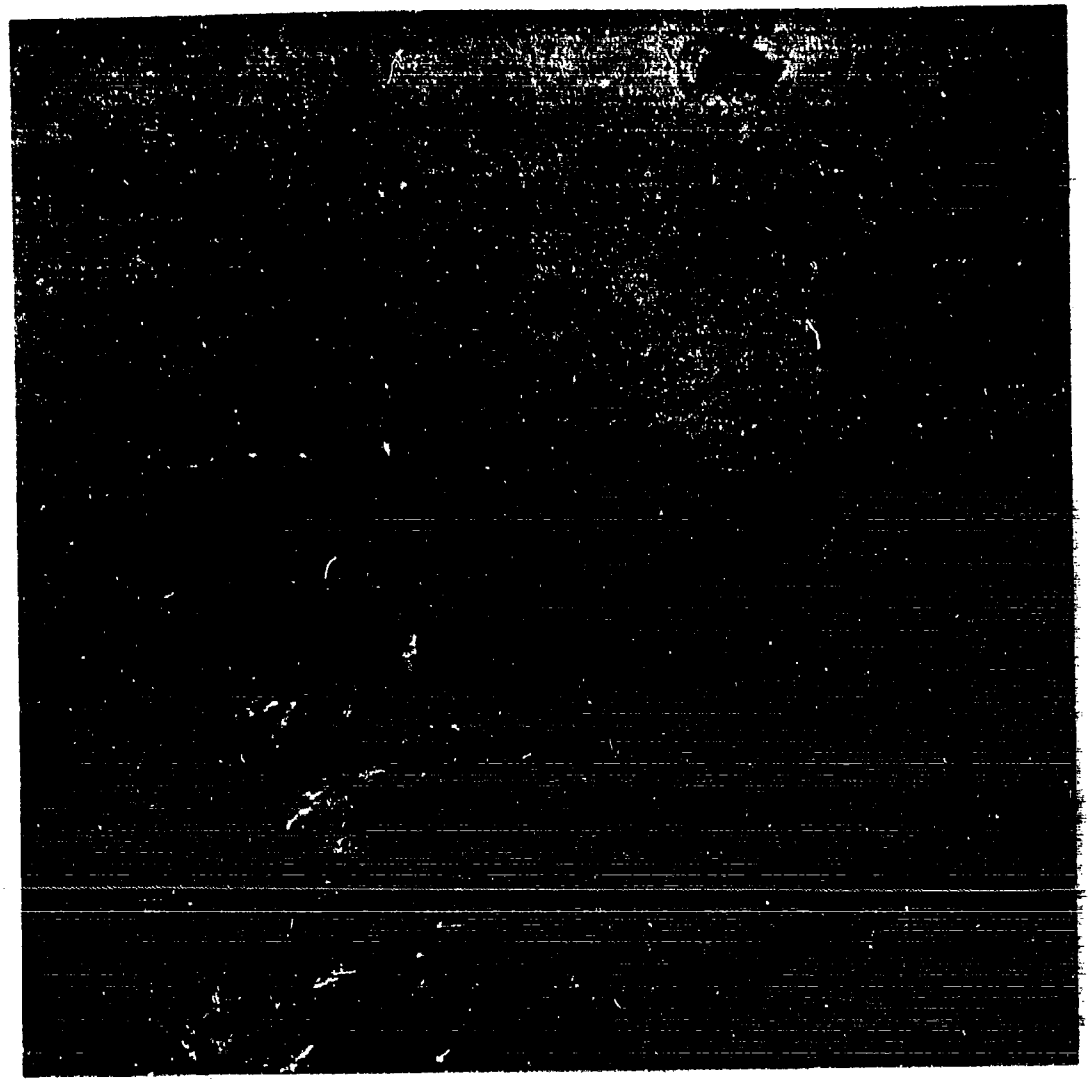

Figure 1. Electron Micrograph of the Purified Organisms of Psittacosis.

IV. DISCUSSION

This investigation was initiated to devise a method for obtaining the organism of psittacosis grown in the yolk sac of the chick embryo free of most of the associated lipids and proteins.

The purification procedure selected may be considered to be effective because: (i) it can be applied to the yolk sac of the chick embryo, which is generally considered to be a rich source of virus; (ii) the procedure removes a large percentage of the extraneous solids; (iii) the partially purified fraction contains a large percentage of the original organisms; (iv) the partially purified organism retains its ability to infect; (v) the procedure is not complex and requires only one centrifugation step. Previously reported methods require multiple steps and have not been primarily concerned with actual over-all recovery of viable organisms. The number of infectious organisms per ml reported here are higher than those previously reported in purified products.

The authors do not suggest the ultimate in purification by this simple process, but believe it to be a significant step in purifying psittacosis organisms from complex suspensions such as yolk sac.

LITERATURE CITED

1. Craigie, J. 1945. Application and control of ethyl-ether-water interface effects to the separation of rickettsiae from yolk sac suspensions. Can. J. Res. E 23:104.

2. Ormsbee, R.A. 1962. A method of purifying Coxiella burnetii and other pathogenic rickettsiae. J. Immunol. 88:100.

3. Comer, J.F., and R.F. Wachter. 1963. Effect of dichlorotetrafluoroethane on the infectivity of virus and rickettsiae sensitive to trichlorotrifluoroethane. Bacteriol. Proc. p. 144.

4. Silverman, R., and P. Fiset. 1963. Purification of psittacosis agent with anion exchange cellulose, "ECTEOLA." Nature 198:699.

5. Sakagami, T., and D.B. Zilversmit. 1962. Dextran sulfate precipitation and ultracentrifugation of lipoproteins from hypocholesterolemic dog serum. J. Lipid Res. 3:111-116.

6. Rivers, T.M., and F.L. Horsfall, Jr. 1959. Viral and rickettsial infections of man. 3rd ed. J.B. Lippincott Co., Philadelphia, Pa. 948 p.

7. Olson, B.J., and C.L. Larson. 1944. An epidemic of severe pneumonitis in bayou region of Louisiana. Public Health Rep. 59:1373-1374.

8. Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall. 1951. Protein measurement with folin phenol reagent. J. Biol. Chem. 193: 265-275.

9. Reed, L.J., and H. Muench. 1938. A simple method of determining fifty per cent endpoints. Amer. J. Hyg. 27:493.